Open access • Journal Article • DOI:10.1093/RPD/NCL495

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Published on: 01 Dec 2006 - Radiation Protection Dosimetry (Oxford University Press)

Topics: Radiation Induced DNA Damage, DNA damage and DNA repair

Radiation induced DNA damage responses

Article (Unspecified)

Radiation Protection Dosimetry (2007), 1 of 4
doi:10.1093/rpd/ncl495

INVITED PAPER

RADIATION-INDUCED DNA DAMAGE RESPONSES

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The amazing feature of ionising radiation (IR) as a DNA damaging agent is the range of lesions it induces. Such lesions include base damage, single strand breaks (SSBs), double strand breaks (DSBs) of varying complexity and DNA cross links. A range of DNA damage response mechanisms operate to help maintain genomic stability in the face of such damage. Such mechanisms include pathways of DNA repair and signal transduction mechanisms. Increasing evidence suggests that these pathways operate co-operatively. In addition, the relative impact of one mechanism over another most probably depends upon the cell cycle phase and tissue type. Here, the distinct damage response pathways are reviewed and the current understanding of the interplay between them is considered. Since DNA DSBs are the major lethal lesion induced by IR, the focus lies in the mechanisms responding to direct or indirectly induced DSBs.

PATHWAYS RESPONDING TO RADIATION-INDUCED DOUBLE STRAND BREAKS AND STALLED REPLICATION FORKS

Non-homologous end-joining

The damage response pathways responding to double strand breaks (DSBs) include pathways of DNA repair and phosphoinositol-3-kinase like kinase (PIKK)-dependent signalling pathways. The major DSB repair pathway is DNA non-homologous end-joining (NHEJ), a process that requires Ku, DNA–PKcs, Xrcc4 and DNA ligase IV as core components. Current models suggest that the heterodimeric Ku protein rapidly binds to double-stranded DNA ends and recruits DNA–PKcs, generating the DNA–PK holoenzyme complex, with activation of its kinase activity. Artemis, a member of the β-lactamase protein family, is also frequently described as an NHEJ component. However, in contrast to defects in other NHEJ proteins, the majority of DNA DSBs are repaired normally in Artemis-defective cells suggesting that it is not a core NHEJ protein. Nonetheless, $\sim$10% of IR-induced DSBs are repaired in an Artemis-dependent manner[(1)]. Since this process requires Artemis nuclease activity, it has been proposed that Artemis is involved in processing a sub-set of damaged ends. Significantly, DSBs induced by the topoisomerase inhibitor, etoposide, do not require Artemis for rejoining supporting the notion that Artemis functions in end-processing since etoposide generated double-stranded ends do not have associated base and sugar damage[(1)]. Artemis is also required for cleaving the hairpin ended DSBs generated during V(D)J recombination, providing evidence for a role in end-processing[(2)].

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Homologous recombination

Homologous recombination (HR) represents another DSB rejoining process, which elegantly uses an undamaged homologue to repair a DSB thereby providing a process capable of achieving high fidelity even if sequence information is lost at the site of the break. In mammalian cells, HR functions primarily in S or early G_2 phase using a sister chromatid as the homologue. Use of a homologous chromosome as a template occurs rarely. An early step in HR is resection of the DNA end to generate a single-stranded region of DNA, which initially becomes coated with the single strand binding protein, RPA. RPA is subsequently replaced by Rad51, which promotes invasion of the template strand. Depending on the precise nature of the process, a Holliday junction may then be generated, followed by branch migration and finally resolution of the Holliday junction. Proteins involved in HR include Rad54, Xrcc2, Xrcc3, Rad51B, Rad51C and Rad51D. Brca2 functions in regulating Rad51 loading onto DNA and Brca1, also, most probably plays a regulatory role in the process[(3,4)].

Damage response signal transduction pathways

The most significant signal transduction pathway responding to DSBs involves ataxia telangiectasia mutated (ATM)[(5)]. Ataxia telangiectasia and Rad3-related protein (ATR), in contrast to ATM, responds to single-stranded regions of DNA which are generated at stalled replication forks[(6)] (Figure 1). ATR may also be activated at single-stranded regions of DNA exposed during processing of certain DNA damages, such as pyrimidine dimers, but such lesions are not generated frequently by IR. Recent evidence has demonstrated that the Mre11/Rad50/Nbs1 (MRN) complex also functions in PIKK signalling. The C-terminus of Nbs1 interacts with ATM

and Nbs1 is required to recruit and activate ATM at the damage site[7]. There is also evidence that the MRN complex has a downstream role in ATM signalling enhancing ATM's retention at the damage site, thereby facilitating its ability to phosphorylate its substrates. In addition, recent evidence suggests that MRN also plays a role in ATR signalling, potentially by enhancing the retention of ATR at the damage site[8]. ATM and ATR activate cell cycle checkpoint arrest and/or apoptosis. Such checkpoints include arrest at the G_1/S boundary, inhibition of late origin firing (intra-S) and prevention of entry into mitosis (G_2/M). In addition, ATR signalling serves to stabilise stalled replication forks. Although ATM and ATR overlap in substrate specificity, there are likely distinctions between them[9]. An early step in both ATM and ATR signalling is phosphorylation of the histone variant, H2AX, generating γ-H2AX[10]. H2AX phosphorylation extends over mega base pair regions of DNA from the break site and can be readily visualised as foci (γ-H2AX foci) using antibodies specific for phosphorylated H2AX. There appears to be a close 1:1 relationship between the number of DSBs and γ-H2AX foci formed, which has provided the basis for an exquisitely sensitive assay to monitor DSB induction[11]. Moreover, the rate of loss of γ-H2AX foci correlates with the rate of DSB repair, providing a sensitive DSB repair assay[1]. Phosphorylated H2AX functions as an important factor, facilitating the retention of additional proteins, such as the MRN complex, MDC1, 53BP1 and Brca1, at the break site[12]. Taken together, the steps of PIKK signalling include sensor proteins, such as the MRN complex for ATM signalling, transducer kinases, such as Chk1 and Chk2 which transduce the signal to downstream effector proteins. Mediator proteins serve to enhance the ability of the PIKKs to phosphorylate their different substrates.

There is also evidence for interplay between PIKK signalling and the two DSB repair processes, HR and NHEJ. The majority (80–90%) of DSBs induced by IR are repaired by NHEJ in an ATM- and ATR-independent manner. Furthermore, ATM-dependent signalling and cell cycle checkpoint arrest occurs efficiently in NHEJ-deficient cells. Thus, NHEJ proteins and ATM are independently recruited to DSBs. However, a subset of DSBs (~10%) requires ATM for their repair[1]. This process requires Artemis, thereby linking ATM signalling to NHEJ. The prevailing evidence suggests that ATM is required for a mechanism of end-processing that involves Artemis nuclease activity, which functions prior to DSB rejoining by NHEJ. Interestingly, Artemis is phosphorylated by ATM *in vivo* although the functional significance of this has not yet been established[1].

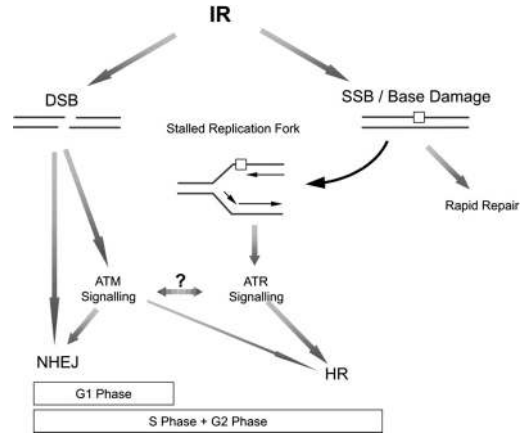

Figure 1. IR induces DSBs, SSBs and base damage. SSBs and base damage are rapidly repaired but can give rise to stalled replication forks. DSBs can be directly repaired by NHEJ. ATM-dependent signalling is activated by DSBs and regulates a component of NHEJ. ATM also activates cell cycle checkpoint arrest. These pathways (except HR) function in all cell cycle stages. ATR is activated by single-stranded regions of DNA generated at stalled replication forks and potentially by resection of DSBs. ATR regulates cell cycle checkpoint arrest and HR. These processes only occur in S and G2 after IR.

HR is also regulated by PIKK-dependent signalling[4]. Since HR functions to repair lesions at replication forks, such regulation is primarily carried out by ATR-dependent signalling. ATR and Chk1-deficient cells are both impaired in HR, which most probably functions at a stage upstream of Rad51 loading[13]. Recently, a phosphorylation site on Brca2 was identified that regulates Rad51 binding in a CDK-dependent manner[4]. The emerging model suggests that HR may be regulated in a cell cycle dependent manner by CDK-dependent phosphorylation of Brca2 with such phosphorylation being sensitive to PIKK-dependent signalling.

ROLES PLAYED BY THE DAMAGE RESPONSE PATHWAYS AFTER IR

A-T cell lines (defective in ATM) and cell lines defective in NHEJ components show exquisite radiosensitivity demonstrating the importance of these pathways in response to IR. Nonetheless, HR-deficient cell lines display modest radiosensitivity[14]. In addition, although ATR-deficient fibroblasts show little radiosensitivity, elevated nuclear fragmentation after IR is observed in ATR-deficient lymphoblastoid cell lines[15,16]. Thus, all four damage response pathways play some role in the response to IR. In the following section, these roles are considered as well as the interplay between them.

Responses functioning in G_1

The majority of cells in an asynchronous human fibroblast population are in a G_0 or G_1 state. Thus, processes that function in G_1 play a determining role in response to damage in such cells. Transformed human cells, mouse embryo fibroblasts (MEFs) and EBV-transformed lymphoblastoid cell lines are more rapidly dividing and have a larger S and G_2 phase population. Thus, processes that function in S and G_2 most probably make a greater contribution to survival and genomic stability in these cell types. Thus, it is important to consider how cell cycle phase may influence the contribution of, and interplay between, the damage response pathways.

The available evidence suggests that HR is regulated in a CDK-dependent manner and is downregulated in G_1[17]. G_1 radiation-induced γ-H2AX foci formation is essentially abolished in cells impaired for ATM and DNA–PK function, strongly suggesting that radiation damage does not activate ATR in G_1 phase. This is consistent with the fact that neither single strand nicks or base damage nor lesions generated during their processing serve to generate the longer regions of single-stranded DNA required for ATR activation. Single-stranded regions of DNA can also be potentially generated by resection of double-stranded DNA ends[17]. However, recent evidence suggests that resection of double-stranded DNA ends fails to occur in G_1. Consequently, NHEJ and ATM-dependent signalling make the major contribution to survival in G_1, consistent with the dramatic radiosensitivity of primary human fibroblasts derived from A-T and LIG4 syndrome patients (which are deficient in DNA ligase IV). A lack of contribution of ATR signalling in G_1 also explains the normal survival response of fibroblasts derived from ATR-Seckel syndrome cell lines, which have low-ATR activity[15].

Since primary fibroblasts do not readily undergo apoptosis, the damage response processes that require consideration are ATM-dependent checkpoint arrest (primarily G_1/S phase arrest), core NHEJ-dependent DSB rejoining and the ATM-dependent component of DSB repair. Interestingly, DSBs repaired in an Artemis-ATM-dependent manner represent those that are normally repaired with slow kinetics, potentially because they represent the more complex or 'dirty-ended' DSBs[1]. Thus, arrest at the G_1/S boundary provides additional time to repair those DSBs repaired in an ATM-dependent manner, providing a clever dual impact of ATM function.

Artemis-defective cells show normal cell cycle checkpoint arrest[1]. Thus, a comparison of the radiosensitivity of Artemis and A-T cell lines is informative in evaluating the contribution of ATM's repair versus the checkpoint functions. Artemis and ATM-defective cells show similar radiosensitivity at doses up to 3 Gy IR, suggesting that ATM's repair function makes a significant contribution to survival. However, loss of the G_1/S checkpoint arrest in repair proficient cells may enhance sensitivity since checkpoint arrest provides the greatest benefit for the slowly repaired DSBs.

Responses functioning in G_2

It is likely that ATM's G_2/M checkpoint function may be particularly important in G_2 due to the short duration of G_2 allowing little time for complete repair before entry into mitosis. The elevated chromosome aberrations observed in G_2-irradiated A-T cells provides evidence for this[18]. Since sister homologues are present in G_2, it is also possible that HR can take place. Furthermore, it is possible that end-resection can take place in G_2, possibly generating single-strand regions of DNA, thereby activating ATR as well as HR (Figure 1).

Responses functioning in S phase

Perhaps the most interesting interplay between the damage response pathways is likely to take place in S phase. Although SSBs and base damage are rapidly repaired, IR induces 20-fold higher levels of SSBs relative to DSBs making them a significant lesion. Replication fork stalling, and hence ATR and HR activation, can occur at such lesions, particularly in rapidly growing cells. However, DSBs induced in S phase cells at non-replication fork sites are likely to activate ATM. It is, therefore, likely that following IR in S phase, ATM and ATR are both activated. Interestingly, although damage response phosphorylation is abolished in A-T cells at early times post-IR, phosphorylation is observed at later times in more rapidly dividing cells, which most probably represents ATR activation as lesions are encountered at the replication fork[19]. This occurs more strikingly in rapidly dividing transformed cells that lack G_1/S checkpoint arrest compared with primary fibroblasts. In addition, the intra-S phase checkpoint is reduced in A-T cells at early times post irradiation, generating the RDS phenotype, but is observed at later times, which again has been proposed to be ATR dependent[20]. Finally, A-T cells in G_2 at the time of irradiation fail to arrest at the G_2/M checkpoint although arrest is observed at later times[21]. Another important function of ATR, in addition to the activation of intra-S and G_2/M checkpoint arrest, is the stabilisation of stalled replication forks, a function which does not appear to overlap with ATM[22]. Interestingly, ATR-Seckel lymphoblastoid cell lines display elevated nuclear fragmentation compared with control cells following

IR possibly explained by a failure to stabilise stalled replication forks[16].

Conclusions

Taken together, the prevailing evidence suggests that, as a consequence of the nature of the damage induced by IR, a range of damage response pathways are called into play. Whilst there is now a good understanding of the mechanism of individual pathways, a future challenge is to understand the complex interplay between them.

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